United States Department of Agriculture
Agricultural Marketing Service, Science & Technology Programs
Microbiological Data Program

SOP NO: MDP-MTH-14		Page 1 of 18
Title: Detection of Shiga-toxin carrying *Escherichia coli* (STEC), serotype O157 and non-O157 in Fresh Produce and Food by Real-Time Polymerase Chain Reaction (Rt-PCR) using the Applied Biosystems® 7500 Fast Instrument with non-O157 STEC Isolation and Identification		
Original	Replaces: None	Effective: 6/1/2012

1. Purpose

To provide a standard procedure for the detection of shiga-toxin carrying *Escherichia coli* (STEC), serotype O157 and non-O157 in commodities analyzed for the USDA AMS Microbiological Data Program (MDP) and isolation and identification of the non-O157 STEC.

2. Scope

This standard operating procedure (SOP) shall be followed by all laboratories conducting microbiological studies for MDP, including support laboratories conducting non-routine activities. This SOP represents minimum MDP requirements and is presented as a general guideline. Each laboratory shall have written procedures that provide specific details concerning how the procedure has been implemented in that laboratory.

3. Principle

STECs are detected by a multiplex real-time PCR assay specific for the shiga-toxins encoding genes, *stx*1 and *stx*2 and the O-antigen polymerase encoding gene, *wzy*, unique to *E. coli* O157 to screen for both O157 and non-O157 serotypes. This method uses Applied Biosystems® 7500 Fast real-time PCR cycler as a platform for the amplification of target genes.

4. Safety

E. coli O157:H7 is a human pathogen with a low infectious dose. Laboratory personnel should utilize Biosafety Level II (BSL-2) practices for microbiological manipulations of known and potential pathogens. A BSL-2 laminar flow biosafety cabinet is recommended for activities with potential for producing aerosols of pathogens. As a standard practice with handling infectious agents, personal protective equipment (PPE) should be used (example: disposable gloves and laboratory coats, or disposable over-garments). Additional respiratory protection (example: face masks) should also be considered for handling infectious agents in dry powder form or large culture volumes. Material Safety Data Sheets (MSDS) should be obtained from manufacturers for media, chemicals and reagents used in the analysis and personnel who will handle the materials should know the location of and have ready access to the MSDS sheets for reference.

5. Outline of Procedures

6. References

- Jinneman KC, Waite-Cusic JG., and Yoshitomi KJ. (2012). Evaluation of shiga toxin-producing *Escherichia coli* (STEC) method for the detection and identification of STEC O104 strains from sprouts. 30:321-328.
- Detection of shiga-toxin *Escherichia coli* (STEC), serotype O157 and non-O157 in food by real-time PCR using the Applied Biosystems® 7500 Fast Instrument – a draft protocol copy provided by the Food and Administration Food Emergency Response Network (FDA FERN) March 7, 2012.
- BAM Online, Chapter 4a: Diarrheagenic *Escherichia coli*. Last updated: 02/2011. Authors: Peter Feng and Stephen Weagant. http://www.fda.gov/Food/ScienceResearch/LaboratoryMethods/BacteriologicalAnalyticalManualBAM/ucm070080.htm - last accessed 03/2012.
- BAM Online, Media Preparation; updated 04/2011 Revision A http://www.fda.gov/Food/ScienceResearch/LaboratoryMethods/BacteriologicalAnalyticalManualBAM/ucm055778.htm - last accessed 03/2012.
- SOP No. MDP-MTH-11: Real-time PCR detection of shiga-toxin producing *Escherichia coli* (STEC), serotype O157 and non-O157 in fresh produce and food with non-O157 isolation and identification Revision 1; Effective date 9/1/2011. USDA Microbiological Data Program (MDP).

- Biosafety in Microbiological and Biomedical Laboratories (BMBL), 5th Edition. http://www.cdc.gov/od/ohs/biosfty/bmbl5/bmbl5toc.htm. U.S. Department of Health and Human Services, Public Health Service, Centers for Disease Control and Prevention and National Institutes of Health:
- SOP MDP-LABOP-02, Sample Receipt, Elution, Pre-enrichment and DNA Extraction
- SOP MDP-DATA-01, Record Keeping and Results Reporting
- SOP MDP-QA-03, Quality Assurance (QA) Controls
- VITEK® Users Manual, bioMérieux

7. Specific Procedures

7.1 Equipment and Materials

- Applied Biosystems® 7500 Fast Real-Time PCR System (SDS version 1.4 with Rapid Finder Express (RFE) v1.1
  - ABI 7500 Fast Plates (AB# 4346906)
  - Optical Adhesive Film for 7500 Fast Plates (AB# 4311971)
  - 8-well strips; alternative to plates (AB# 4358293)
  - Optical caps for 8-well strips (AB# 4323032)
  - Appropriate ABI 7500 Fast Plate Holder (specific for 96-well tray or 8-strip well tubes)
- Vortex mixer
- Microcentrifuge tubes, 1.5 ml
- Micropipetors and aerosol resistant tips (1.0 – 1000 µl capacity)
- Incubators: 35 ± 2ºC, 42 ± 2ºC and 44 ± 2ºC
- VITEK® System or VITEK® 2 Compact System, bioMérieux
- VITEK® GNI+ Card or GN cards, bioMérieux

7.2 Media and Reagents

- Molecular Grade H_2O
- Express qPCR Supermix Universal Taq (Invitrogen, Carlsbad, CA. #11785200)
- TaqMan® Exogenous Internal Positive Control (Applied Biosystems, Carlsbad, CA. (#4308323)
- 10 µM Working Solution each primer listed in Table 1. Stock and Working solutions can be prepared from commercially synthesized primers with basic desalt purification

(Fisher/Genosys or equivalent) by rehydrating with sterile distilled water to appropriate concentrations. Store at –20°C to -70°C until use.

- 10 µM Working Solution each probe listed in Table 1. Stock and Working solutions can be prepared from commercially synthesized probes with RP HPLC purification. Working solutions of probes can be aliquoted into small portions stored frozen (-20 to -70°C) until use.
- TP Broth
- Chromogenic agar plates (Example: DRG Chromogenic agar, etc.)
- TCSMAC (Tellurite-Cefixime Sorbitol MacConkey Agar) plates
- L-EMB Agar
- MacConkey Agar
- BA (Blood Agar) plates
- LST broth

7.3 List of Controls (Specific strains are listed in SOP MDP-QA-03)

7.3.1 Carry all cultural controls from all screening methods previously completed through this entire procedure. Refer to SOP MDP-LABOP-02 for control setup. If any of the controls fail to yield a satisfactory result refer to SOP MDP-QA-03.

- No-template Control: Transfer 25 µl of Master Mix and 5 µl of PCR grade water
- Negative Culture Control: DNA from MDP-017
- Positive Cultural Control: DNA from MDP-004
- Positive Produce Control: DNA from inoculated produce culture control (MDP-004) from SOP MDP LABOP-02
- Media Control
- Positive DNA Control: MDP-019: grow culture and extract DNA prior to assay setup

7.4 Template, Primers/Probe and Master Mix Preparations

7.4.1 Use extracted DNA from overnight UPB enriched samples from SOP MDP-LABOP-02.

7.4.2 Table 1 lists primer and probe details and table 2 provides their concentrations

7.4.3 Each reaction without the template DNA shall be 25 uL. Using Table 2 reconstitute the primers, probes, IPC (DNA and probe), ROX dye, Supermix and sterile molecular grade water to make up the volume to the desired number of reactions. Mix.

7.4.4 Dispense 25uL of the above mix to each PCR tube.

7.4.5 Transfer 5 uL of sample DNA to each reaction tube.

7.4.6 For positive and negative controls add 5uL of appropriate DNA samples and 5 uL of water in place of DNA sample for no-template sample (see section 7.3 List of Controls)

Note: Cap tubes or seal plate carefully to avoid well-to-well contact.

- *Spin 8-strip tubes or 96-well plate in appropriate instrument to bring contents to bottom of tube.*
- *Place tubes into ABI 7500 Fast Instrument.*

Note: Ensure appropriate tray is used for 96-well plate or 8-strip well.

Table 1: Primer/probe sequences for detection of STEC for use on AB 7500Fast platform

Primers[1]	**GenBank #**	**Bases**	**5'→ 3' Sequence**
stx1F934	M19473	26	gTg gCA TTA ATA CTg AAT TgT CAT CA
stx1R1042	M19473	21	gCg TAA TCC CAC ggA CTC TTC
stx2F1218	X07865	24	gAT gTT TAT ggC ggT TTT ATT TgC
stx2R1300	X07865	26	Tgg AAA ACT CAA TTT TAC CTT TAg CA
wzyF1831	AF061251	24	CTC gAT AAA TTg CgC ATT CTA TTC
wzyR1936	AF061251	23	CAA TAC ggA gAg AAA Agg ACC AA
Probes[1]			
stx1P990	M19473	31	Cy5-TgA TgA gTT TCC TTC TAT gTg TCC ggC AgA T-BHQ2
stx2P1249	X07865	25	TAMRA-TCT gTT AAT gCA ATg gCg gCg gAT T- BHQ2
wzyP1881	AF305917	15	6FAM - ACT TAg Tgg CTg ggA ATg CAT Cgg C – BHQ1

[1]Primer/Probe name composed of target gene (*stx*1, *stx*2 or *wzy*), forward primer (F), reverse primer (R) or probe (P), 5' base position of oligonucleotide in the respective gene sequence specified in column 2.

- Internal Positive Control:
  - The Internal Positive Control (IPC) is included in every assay using the TaqMan Internal Positive Control (Invitrogen #4308323).
  - The IPC is detected by a PCR product being detected in the VIC channel (Ch.2) at approximately 25-30 cycles. Note the IPC may or may not be detected in any reaction, especially when one or more gene targets are amplified.

Table 2. AB 7500 Fast STEC Reaction Components

Volume/rxn	Component	Final Conc.
qsi to 30 µl	Sterile Distilled Water	
0.75 µl	Primer stx1F934 (10 µM Work Solution)	0.25 µM
0.75 µl	Primer stx1R1042 (10 µM Work Solution)	0.25 µM
0.75 µl	Primer stx2F1218 (10 µM Work Solution)	0.25 µM
0.75 µl	Primer stx2R1300 (10 µM Work Solution)	0.25 µM
0.90 µl	Primer wzyF1831 (10 µM Work Solution)	0.30 µM
0.90 µl	Primer wzyR1936 (10 µM Work Solution)	0.30 µM
0.60 µl	Probe stx1P990 Cy5 (10 µM Work Solution)	0.20 µM
0.45 µl	Probe stx2P1249 TAMRA (10 µM Work Solution)	0.15 µM
0.45 µl	Probe wzyP1881 FAM (10 µM Work Solution)	0.15 µM
3.00 µl	Internal Positive Control Primer/Probe Mix[1]	
0.60 µl	IPC DNA[1]	
15.0 µl	Express qPCR Supermix Universal[2]	
0.06 µl	ROX dye[2]	
0.04 µl	Molecular Grade Water	
5.00 µl	Template (Sample or control)	
30.0 µl	Total Reaction Volume	

[1] Included in TaqMan® Exogenous Internal Positive Control Kit (Applied Biosystems, Carlsbad, CA. #4308323)

[2] Included in Express qPCR Supermix Universal Taq (Invitrogen, Carlsbad, CA.#11785200)

Table 3: Mixing of components to setup PCR reactions

Component (10 uM working solution)	Volume per rxn (uL)	Volume per 20 rxns (uL)
Primers		
Primer stx1F934	0.75	15
Primer stx1R1042	0.75	15
Primer stx2F1218	0.75	15
Primer stx2R1300	0.75	15
Primer wzyF1831	0.90	18
Primer wzyR1936	0.90	18
Probes		
Probe stx1P990 Cy5	0.60	12
Probe stx2P1249 TAMRA	0.45	9
Probe wzyP1881 FAM	0.45	9
Controls		
IPC	3.0	60
IPC DNA	0.6	12
ROX dye	0.06	1.2
Super Mix Universal		
Express qPCR Supermix Universal	15	300
Water		
Sterile molecular grade water	0.04	0.8
Distribute 25 uL each in 20 tubes		
DNA sample per PCR tube	5	
Total Reaction volume per PCR tube	30	

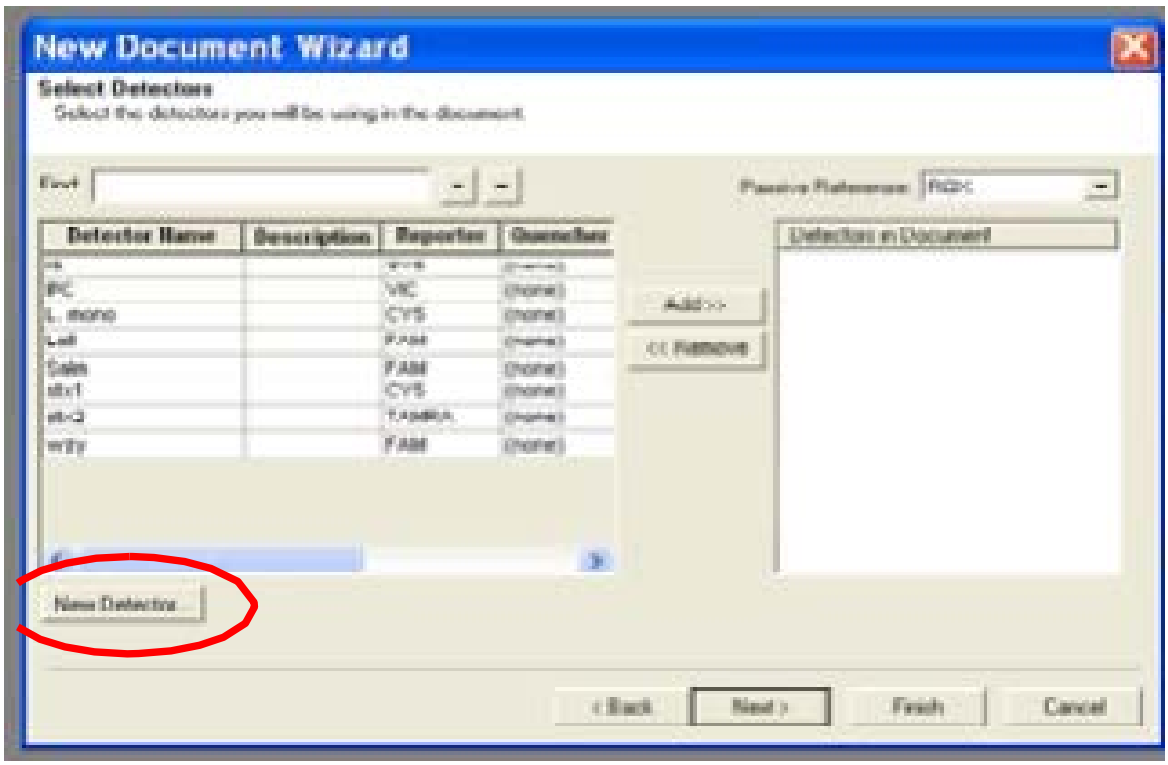
7.5 Real-time PCR Analysis: On the ABI 7500 Fast (SDS ver. 1.4)

7.5.1 Create run on ABI 7500 Fast (SDS ver. 1.4).

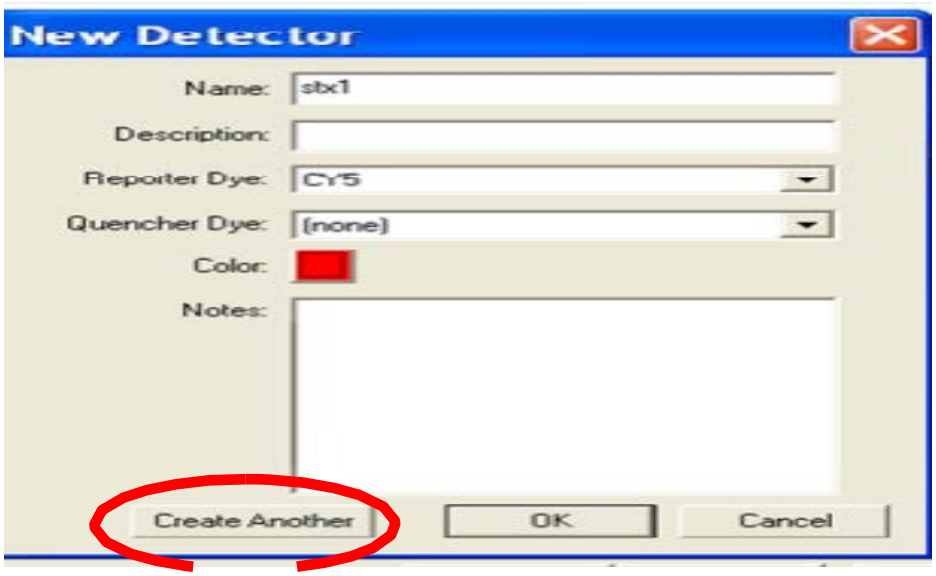
- Click on Create New Document.
- In the New Document Wizard, define the document using the default settings and click "Finish" when complete.

  Assay: Standard Curve (Absolute Quantitation)
  Container: 96 Well – Clear
  Template: Blank Document
  Run Mode: Fast 7500
  Operator: Follow local laboratory guidance
  Comments: Follow local laboratory guidance
  Plate Name: Follow local laboratory guidance

- In the new document wizard, select appropriate detectors for the *E. coli* O157/STEC assay. To add detectors/targets, select the "New Detector…" button.



- In the new document wizard, select appropriate detectors for the *E. coli* O157/STEC assay. To add detectors/targets, select the "New Detector…" button.
- Complete information about the New Detector as follows (other fields can remain empty or as default):
  Name: stx1
  Reporter Dye: Cy5
  Quencher Dye: (none)
  Color: Red

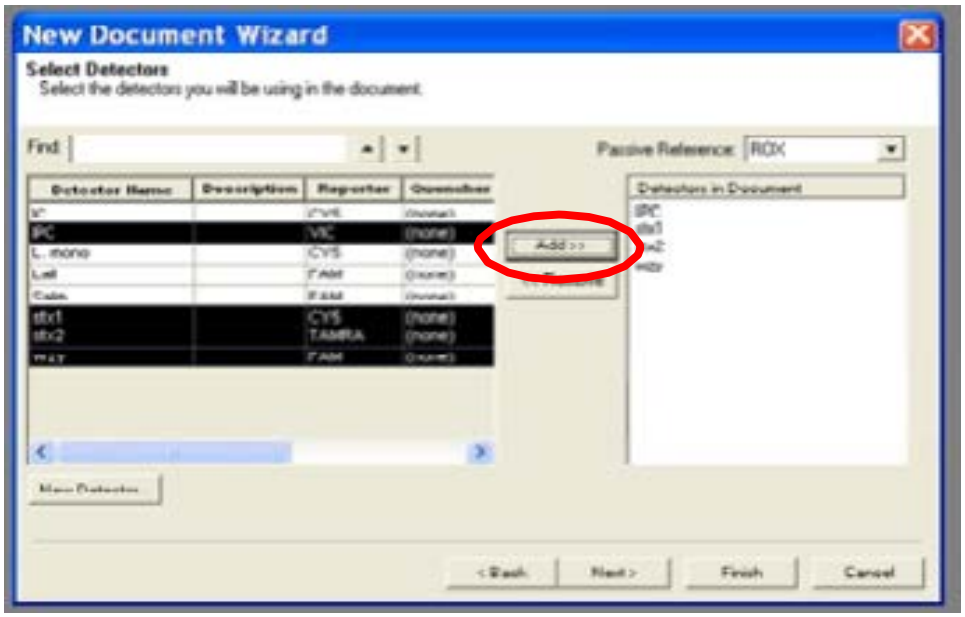


- Add a new detector by clicking on the "Create Another" button.
- Define subsequent detectors until you have completed the following set:

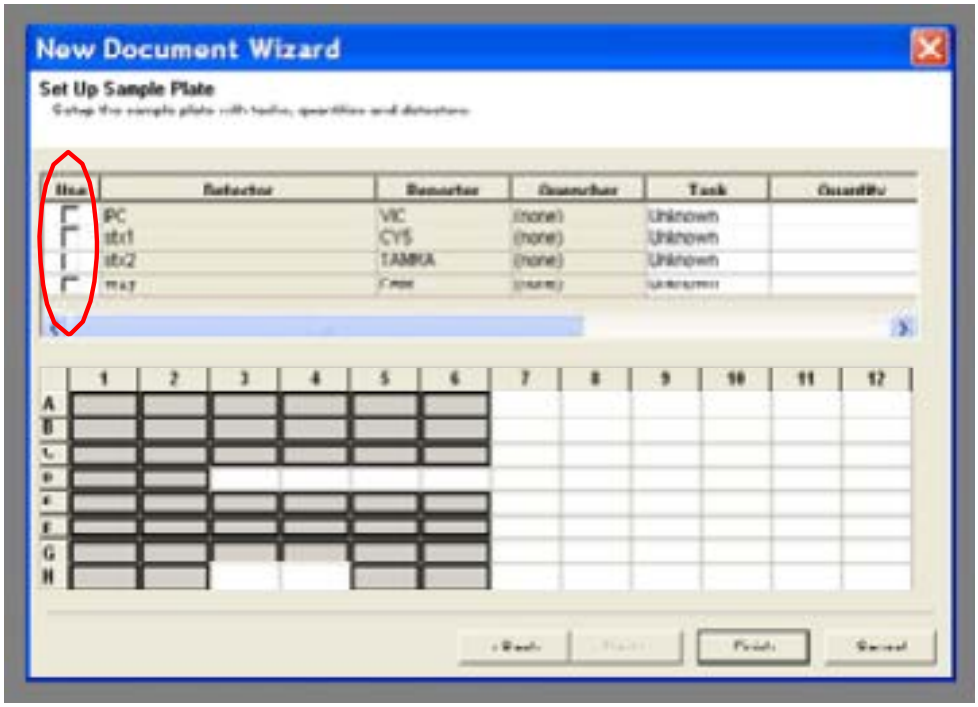
Target Name	Reporter	Color (below or user option)
*stx*1	Cy5	Red
*stx*2	TAMRA	Blue
wzy	FAM	Green
IPC	VIC	Black

- After adding the final detector, click the "OK" button.

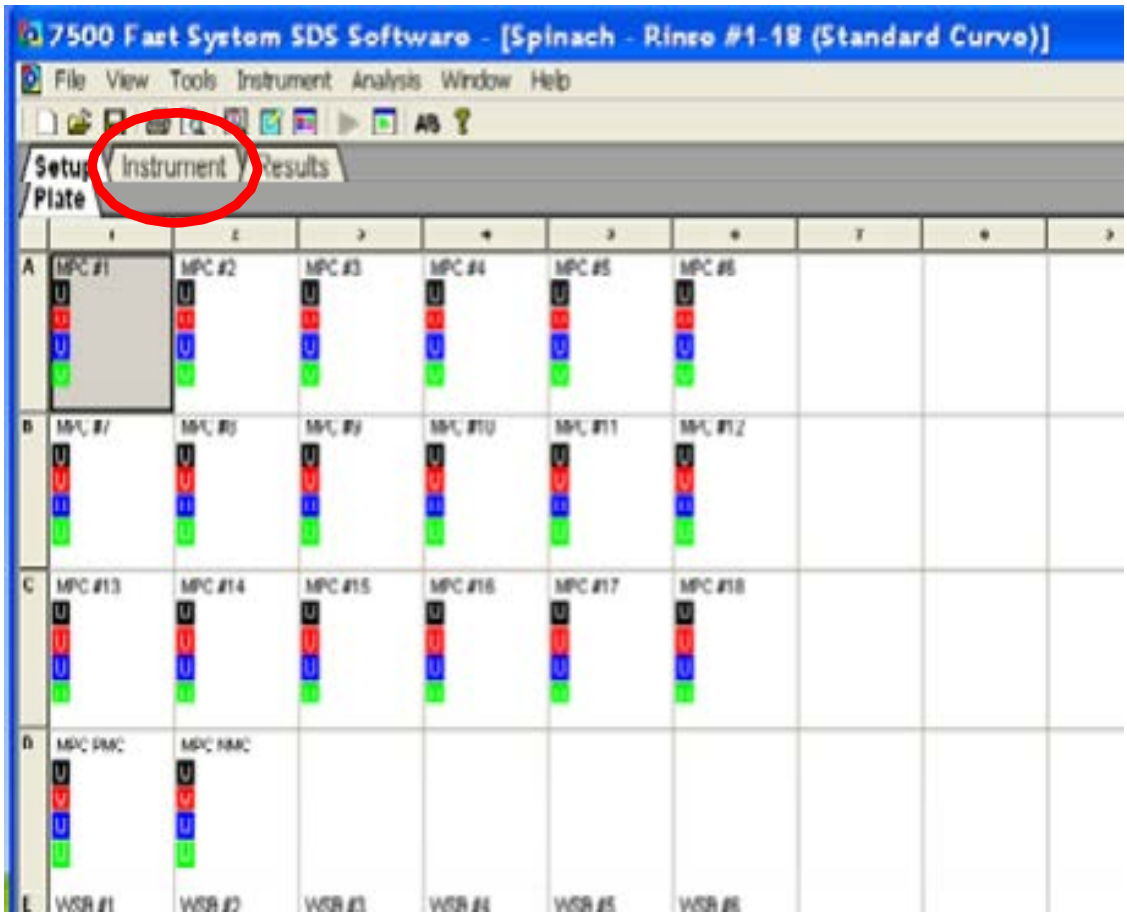
- From the Select Detectors window, highlight and add the appropriate detectors/reporters for the *E. coli* O157/STEC assay: IPC/VIC; stx1/CY5, stx2/TAMRA, and wzy/FAM.



- Select ROX as the Passive Reference dye and click “Finish”.
- To set up the sample plate, highlight wells to be used and select detectors.



- Click "Finish" after adding detectors.
- Highlight sample well and type in sample information.
- Once sample information is entered, click on "Instrument" tab.

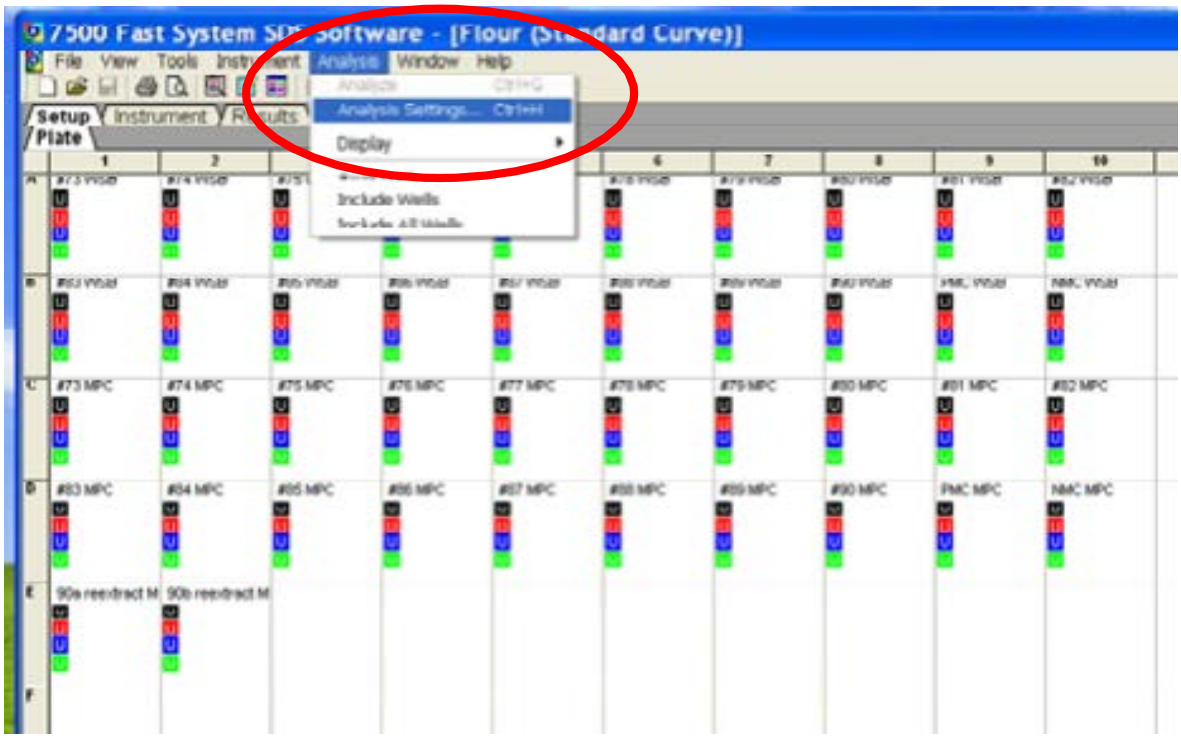


- Change the instrument settings for the *E. coli* O157/STEC assay to the following:
    - Stage 1: 95.0ºC; 1 min
    - Stage 2: 40 cycles
        - Step 1: 94.0ºC; 10 seconds
        - Step 2: 63.0ºC; 40 seconds
    - Sample Volume: 30 µL
    - Run Mode: Fast 7500
    - Data Collection: Stage 2, Step 2 (63.0@0:40)
- Once run settings are finalized, click the "Start" button.
- When prompted, click the "Save and Continue" button.
- Save SDS file as per as per local laboratory protocol. Run time is approximately 1 h.

Note: There are a variety of ways to view/report Cycle Threshold (Ct) values. Follow local laboratory procedures if provided. The analysis settings applied to the run are more important.

7.5.2 Viewing results on the ABI 7500 Fast.

- From the Results Plate window, select "Analysis" from the dropdown menu, followed by "Analysis Settings".



- Set analysis settings for all detectors to Manual Threshold of **0.05**. Check that **Manual Baseline** is set to cyclers **3** and **15** and click the "OK" button.
- Analyze the run by clicking "Analyze" under the "Analysis" menu.
- To view the Ct results on the Results Plate, click on the "Analysis" menu.
- Then click "Display" and select "Ct".
- Record Ct values for respective targets in the assay.
- Other options are available to view Ct's and Graphs under the Results tab.

7. 6. Interpretation of Results

7.6.1 A preliminary positive result for STEC is indicated by DNA detected for a respective gene target (*stx*-1, *stx*-2, *wzy*).

7.6.1.1 If a pooled sample is positive for any one of the targets, extract DNA from individual samples as per SOP MDP-LABOP-02.

7.6.1.2 Setup PCR reactions for the previously extracted pooled and the individual samples as per 7.5 Real-time PCR Analysis section of this SOP. Also, run same samples following SOP MDP MTH-12.

7.6.1.3 Samples that show positive *stx*-1 or *stx*-2 (and are negative for MDP MTH-12) are considered suspect positive for non-O157 STECs and shall be culturally confirmed as per section 7.7 in this SOP.

7.6.1.4 Samples that show positive *wzy* results (regardless of *stx*-1 or *stx*-2 results) by SOP MDP MTH-14 and/or positive results by SOP MDP-MTH-12 are considered presumptive positive for *E. coli* O157:H7 and shall be confirmed according to SOP MDP MTH-06.

7.6.2 A negative result for STEC is indicated when no DNA is detected for a respective gene target (*stx*-1, *stx*-2, *wzy*).

7.6.3 Quality Control Results

- A positive test result occurs when a Cycle Threshold (Ct) value is achieved within the specified range as a result of primary fluorescent signal crossing set threshold value.
- A negative test result occurs when there is no Ct value as a result of primary fluorescent signal not crossing set threshold value.
- Ensure Positive PCR control is positive for all three gene targets (*stx*1, *stx*2 and *wzy*). It is not essential for the Internal Positive Control to be positive in the Positive PCR control (as long as other targets are detected), but it can be present.
- Negative PCR control should be negative for all three gene targets **AND** positive for the Internal Positive Control target.
- Due to the complex nature of produce samples and the sample to sample variations Internal Positive Control may not result in a Ct. The positive, negative and no-template controls should have worked as expected.

7.7 Isolation and Identification of non-O157 STEC

Laboratories are encouraged to use best professional judgment and experience to obtain a pure culture of the target organism. Isolation and identification can be carried out from the pooled sample and the individual sample that tested positive.

7.7.1 For each STEC positive pooled sample (except for alfalfa sprouts), transfer 10 mL of pooled UPB enriched sample to 90 mL TP broth and incubate at 44 ± 2°C for 18-24 hours.

Note: 10 mL of the pooled sample is left over and if required can be used for additional analyses (ex: IMS of the pooled UPB if sample is positive on MDP MTH-12.)

7.7.2 In addition, transfer 25 mL of each of the positive individual UPB enriched samples into 225 mL of TP broth and incubate at 44 ± 2°C for 18-24 hours. For sprouts, transfer 25 mL of the individual positive sample accordingly.

7.7.3 Following incubation, from the enrichment broths, streak duplicate sets of plates of chromogenic agar, L-EMB, and MacConkey plates. Incubate one set of plates overnight at 35 ± 2°C and incubate the other set of plates overnight at 42 ± 2°C.

Note: Use professional judgment and experience in deciding the number of plates needed for picking minimum number of 20 isolated colonies

7.7.4 Examine plates and pick at least 20 typical isolated colonies or swipes from any of the selective agar plates (chromogenic, L-EMB, and MacConkey) to appropriate media (e.g., LST, BHI, etc.). It is advised to pool multiples of isolates and screen on Rt-PCR to determine what isolate(s) that are positive (e.g., pick 20 colonies and pool into 2 groups of 10; example: if you have 20 LST samples, split into two groups containing 10 each) and perform DNA extraction according to SOP MDP-LABOP-02 and perform Rt-PCR. ***Document procedures used accordingly.***

Note: Use professional judgment and experience in deciding the maximum number of colonies required for screening which depends on the extent of contamination, the background microflora level, type of commodity and post harvest handling.

Typical Colony Characteristics of pathogenic *E. coli*	
Medium	**Colony Characteristics**
MacConkey	Red to pink
L-EMB	Blue-black and green w/metallic sheen
Chromogenic agar	refer to manufacturer's user guide

7.7.5 Identify the individual Rt-PCR positive isolate. Streak the isolate to selective agar plates and BA for isolation. Incubate overnight at 35 ± 2°C.

7.7.6 Examine plates and if selective agar plates contain typical colonies, perform VITEK® using growth off BA or other appropriate plating media. (If VITEK® identifies the organism as a possible *E. coli* O157:H7, further identification based on cultural tests and serotype is required as per SOP-MDP-MTH-06. MPO shall be notified per SOP MDP-DATA-01.) Pick 3-5 individual typical colonies to LST or non-specific rich broth.

7.7.7 Repeat Rt-PCR on 3 isolates. Choose one isolate that is identified as *E. coli* and possesses toxin gene(s) for archiving and shipping according to SOP MDP-SHIP-03.

7.8 Tryptone Phosphate (TP) Broth - Autoclave for 15 minutes at 121ºC. Final pH should be 7.0 ± 0.2.

Tryptone	20.0 g
K_2HPO_4	2.0 g
KH_2PO_4	2.0 g
NaCl	5.0 g
Tween 80	1.5 mL
Deionized Water	1.0 L

7.9 Reporting - A "preliminary STEC" is defined as an isolated organism that is shown by Rt-PCR to possess the DNA sequences associated with the stx1 and/or stx2 genes and to NOT possess the *E. coli* O157:H7 DNA sequence (secondary analysis per MDP-MTH-12). The isolate(s) slants are to be shipped to the repository (per MDP-SHIP-03 SOP) for archival and/or additional testing. If additional testing is performed by an outside agency, upon receipt of PFGE/serological results, MPO will provide test data to repository and originating laboratory. Report results according to SOP MDP-DATA-01.

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